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13. ABSTRACT (Maximum 200 Words) The existence of prostate stem cell capable of giving rise to all the epithelial lineages present in the adult prostate is very controversial. Understanding the stages of cell differentiation in normal prostate epithelium is essential for the identification of the cell type(s) involved in prostate carcinogenesis. The p53-homologue p63 is selectively expressed in the basal cell compartment of a variety of epithelial tissues and p63 deficient mice show severe defects in the development of epithelial organs, including agenesis of the prostate. These findings suggest that p63 is required to maintain a prostate stem cell population. In order to test this hypothesis we will first study p63 expression in the various stages of prostate development in wild type mice by both immunohistochemistry and in situ hybridization (Specific Aim 1). We will also construct chimeric mice by injecting p63 ^{+/+} β -galactosidase positive ES cells into p63 ^{-/-} blastocysts (Specific Aim 2) and then analyze the relative contribution of p63 ^{+/+} and p63 ^{-/-} cells to the prostatic epithelium. In the event in which both basal and secretory cells require p63 for development, the results will indicate that both compartments originate from a common p63-positive stem cell.				
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INTRODUCTION

One substantial limitation in understanding the molecular events that lead to prostate cancer is that the cell type undergoing neoplastic transformation in the prostate is unknown. Moreover, although three major cell types have been identified within the prostate epithelium, the hierarchical relation between them remains obscure and the existence of prostate stem cells is uncertain (1-6). It appears clear that unraveling the epithelial hierarchy in the normal prostate epithelium has important implications in identifying the cell of origin of prostate carcinoma and its pathogenetic mechanisms. The aim of this proposal is to identify prostate stem cells.

The p53 homologue p63 is selectively expressed in the basal cell compartment of the several epithelia, including the prostate. p63 knock-out mice show severe defects in the development of epithelial organs, including the agenesis of all squamous epithelia, breasts, salivary glands and lachrymal glands (7,8). We have recently demonstrated that p63 knock-out mice do not develop the prostate (9). These findings imply that during embryogenesis p63 is required to maintain an epithelial cell population that plays a crucial role in prostate morphogenesis. Two main hypotheses can explain the defect in prostate development in p63^{-/-} mice: 1) p63 is essential for maintaining a prostate epithelial stem cell population that generates both basal and secretory cells 2) p63 is essential for maintaining prostate basal cells which do not represent prostate stem cells but are essential for prostate development. In order to test these hypotheses we are constructing chimeric mice by injecting p63^{+/+} β -galactosidase positive ES cells into p63^{-/-} blastocysts. If, as expected, p63^{+/+} ES cells abrogate the defect in prostate development, we will analyze the relative contribution of p63^{+/+} and p63^{-/-} cells to the prostatic epithelium in rescued chimeric mice. This proposal represents a unique approach to resolve the long-standing controversy on the role of prostate basal cells as stem cells. This chimeric model, if successful, may be applied as an innovative approach to study prostate development and neoplastic transformation in vivo. By utilizing genetically altered β -galactosidase positive ES cells (e.g. Rb^{-/-} or p53^{-/-}) it will be possible to generate mice that carry specific genetic alterations targeted to prostate epithelial cells and investigate their role in tumorigenesis and tumor progression.

BODY

Research accomplishments based on the approved Statement of Work

Aim 1. To assess the distribution of p63 positive cells in the developing prostate.

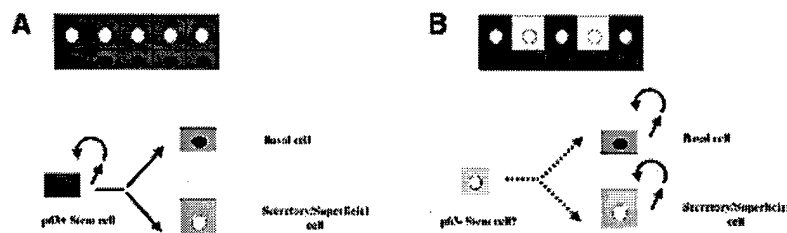
The goal of this specific aim is to confirm that the absence of p63 causes arrest in prostate morphogenesis at the stage of budding from the urogenital sinus by comparing prostate development in wild type and p63^{-/-} mice with a detailed morphologic analysis. In addition, by utilizing immunohistochemistry and in situ hybridization, I plan to determine if all cells in the early prostatic buds of wild type mice are p63 positive.

In year 2002, we performed immunohistochemistry for p63 in the urogenital sinus of 10 wild-type male embryos at 18dpc. Our results demonstrate that during this early stage of prostate development, all the cells in the buds are p63 positive. The levels of expression are higher in the cells localized at the periphery of the buds (i.e. the cells in contact with the extracellular matrix).

In situ hybridization experiments are still under optimization. Skin and prostate tissue sections from wild type mice are utilized as positive controls.

Aim 2: To determine which cell compartment(s) require(s) p63 expression for normal prostate development in the mouse.

In order to assess whether or not the p63 positive epithelial cells in the developing prostate represent prostate stem cells that are able to differentiate into both basal and secretory cells, I am utilizing a chimeric mouse system. The aim of this project is to abrogate the defect in prostate development associated with p63 deficiency by injecting p63^{+/+} ROSA26 (β-gal positive) ES cells into p63^{-/-} blastocysts. In this animal model, information regarding the hierarchical relationship between prostate p63 positive (basal) and p63 negative (secretory) cells can be obtained by assessing the relative contribution of p63^{+/+} and p63^{-/-} cells to the prostate epithelium.



Two main outcomes are predicted. In the first scenario (Fig. 1a), both basal and secretory cell compartments consist exclusively of p63^{+/+} (β-gal positive) cells. This result would imply that both cell compartments require p63 for development and, therefore, derive from a common p63 positive basal stem cell. The second possibility is that p63^{-/-} (β-gal negative) cells contribute to the secretory cell compartment (Fig. 1b).

This outcome would lead to the conclusion that p63 positive cells are not the progenitors of secretory cells. In this case, p63 positive and p63 negative cells either represent completely independent cell lineages or derive from a common p63 negative stem cell.

Generation of chimeric mice

Experimental design: Fifteen to twenty p63^{+/-} female mice were superovulated and mated to p63^{+/-} male mice. ~140 blastocysts were isolated from 12-15 plugged females. 3.5 dpc blastocysts were injected with ROSA26 ES cells and transferred to 10 foster mothers.

This experiment was designed to analyze an extensive number of chimeric embryos by sacrificing the foster mothers at 18.5dpc. This was done in order to unequivocally identify the phenotype of the chimeras derived from p63^{-/-} blastocysts, which are expected to represent 25% of the embryos. The recovery of the mice before birth allowed us to analyze the entire litter and prevent the mothers from killing un-rescued or partially rescued p63^{-/-} newborns. We decided to sacrifice the embryos at 18.5dpc because prostate buds can be already identified at this stage of development. 56 embryos were generated and fully analyzed in this experiment.

Additional blastocyst complementation experiments were performed as described. In these experiments, a total 140 chimeras were generated and analyzed at 7 weeks of age, when the prostate epithelium is fully developed.

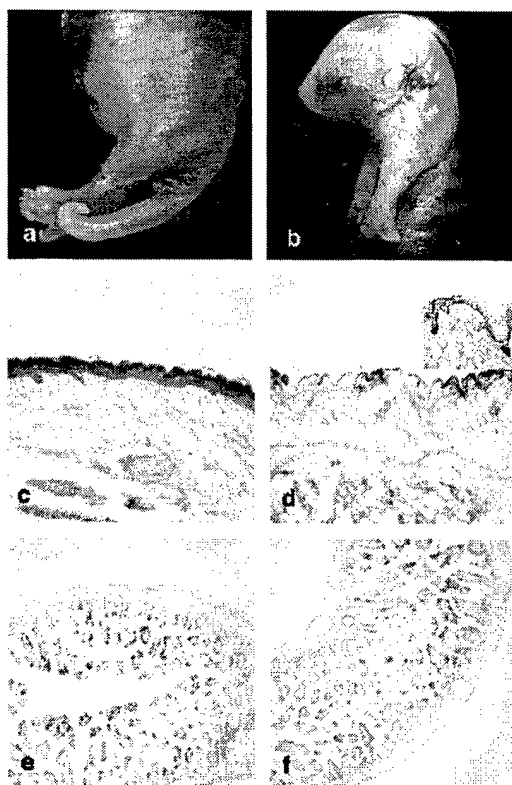
Analysis of chimeric mice

Fifty-six chimeras were generated and analyzed at 18.5dpc. In keeping with the male genotype of the ROSA26 ES cells, 90% of the embryos were males. Fifty embryos appeared normal at external examination and four embryos presented the characteristic p63^{-/-} phenotype. Importantly, two chimeras showed several abnormalities indicating that they represented p63^{-/-} embryos in which limb and skin defects were partially rescued by the injection of the p63^{+/+} ES cells. Indeed, they were characterized by a variety of defects of both forelimbs and hindlimbs, ranging from severe limb truncation to split appearance of the paw (Fig.2a, b). Intriguingly, this latter defect closely resembled the ectrodactyly observed in patients affected by the EEC syndrome, which is caused by heterozygous mutations in the p63 gene.

In addition, these two chimeras presented localized areas of skin ulceration. Histological examination of these areas showed absence of the epidermal lining and epidermal appendages, as typically observed in the p63^{-/-} mice (not shown).

The usefulness of this animal model in elucidating the hierarchical relationship between p63 positive and p63 negative cells in epithelial structures relies on the demonstration that p63 function is cell-autonomous. To achieve this goal, we assessed the relative contribution of β -gal positive (p63^{+/+}) cells versus the β -gal negative cells to the chimeric embryos by performing X-gal histochemistry. The level of chimerism (% of β -gal positive cells) was determined both in organs that require p63 for development (e.g. the epidermis) and in organs/tissues that are p63-independent (e.g. the intestinal

epithelium and cartilage). Most chimeras (79%) showed similar level of chimerism in the



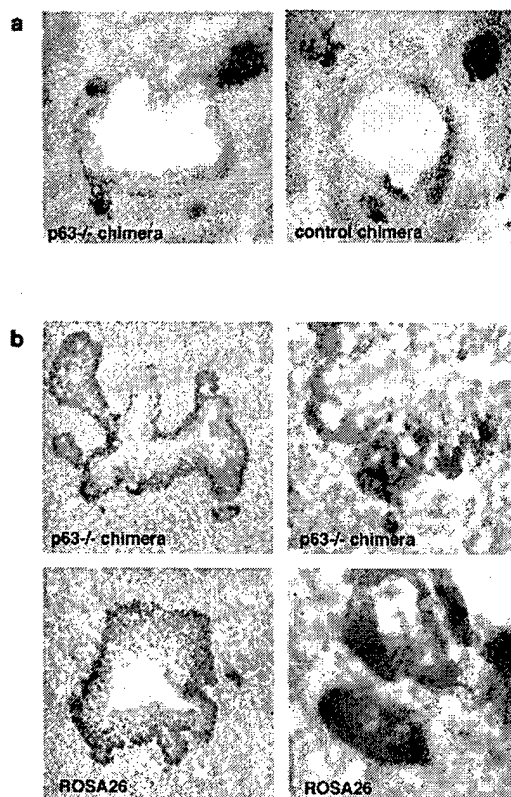
epidermis, the intestinal epithelium, and cartilage (Fig. 2d, f). The level of chimerism ranged from 0 to 100% in the various animals. In order to test if any of these chimeras represented $p63^{-/-}$ blastocysts in which the $p63^{+/+}$ ROSA26 cells had abolished the developmental defects through a non-cell-autonomous $p63$ function, $p63$ immunohistochemistry was performed on skin sections previously stained with X-gal. We were able to detect $p63$ expression in the β -gal negative cells of the epidermis and thus demonstrate that none of these mice derived from the $p63^{-/-}$ blastocysts (Fig. 2d, inset). More importantly, the partially rescued $p63^{-/-}$ embryos were characterized by a distinctive pattern of X-gal staining. In these mice, the squamous epithelia, including the epidermis, were exclusively populated by β -gal positive ($p63^{+/+}$) cells (Fig. 2c). In contrast, the level of chimerism in the intestinal epithelium and cartilage ranged from 30% to 45% (Fig 2e). In summary,

the consistent absence of $p63^{-/-}$ cells in the squamous epithelia of the rescued $p63^{-/-}$ mice unequivocally demonstrated that $p63$ is required in a cell-autonomous manner for the development of epithelial tissues.

Interestingly, the pattern of X-gal staining observed in the partially rescued $p63^{-/-}$ mice was also detected in six additional chimeras that did not show any developmental abnormalities at external examination. This indicated that these chimeras corresponded to $p63^{-/-}$ embryos in which the injection of $p63^{+/+}$ ES cells had lead to the complete rescue of both limb and skin defects. In keeping with this finding, the total number of embryos derived from $p63^{-/-}$ blastocysts represented 21% of all the chimeras. This percentage is very close to rate of $p63^{-/-}$ blastocysts expected from $p63^{+/-}$ crosses (25%).

We previously showed that the $p63^{-/-}$ mice present abnormalities in the urogenital tract, including the absence of prostate buds and the presence of a pseudostratified columnar epithelium replacing the stratified epithelium that normally lines the urogenital sinus (UGS) (ref). We analyzed the UGS of the chimeric embryos to check if the prostate defects of the $p63^{-/-}$ mice had been rescued by the injection of the $p63^{+/+}$ ES cells. Since both the prostate buds and the urogenital sinus epithelium at 18.5 dpc express cytokeratin 5 (CK5) and cytokeratin 14 (CK14), we utilized immunohistochemistry for CK5 and CK14 to reliably identify prostate buds in frozen tissue sections. As expected, no prostate buds were detected in the embryos presenting the characteristic $p63^{-/-}$ phenotype (not rescued). The pseudostratified columnar

epithelium lining the UGS of these embryos showed complete absence of CK5 and CK14 expression (not shown) confirming that p63 is required for normal development of the UGS epithelium. Importantly, prostate buds could be identified in seven of the eight p63^{-/-} rescued male mice. Remarkably, these buds were exclusively populated by



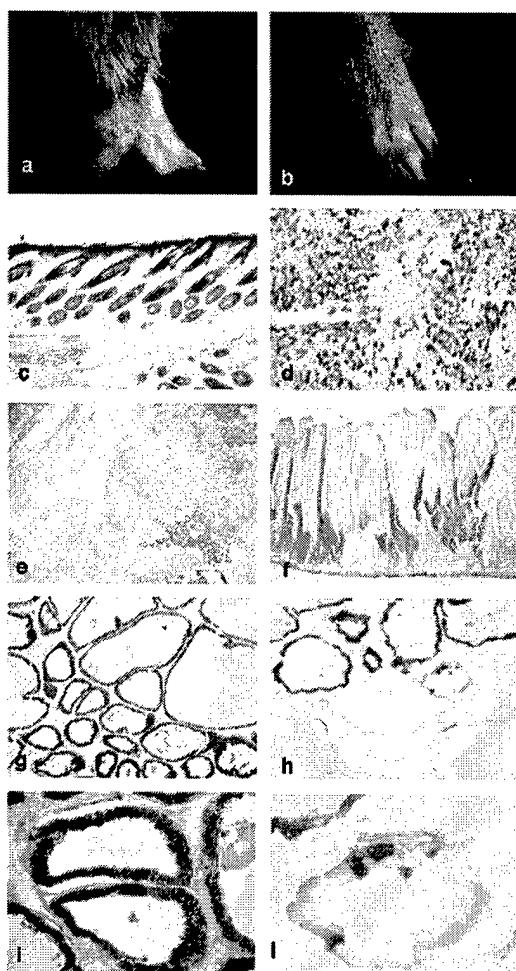
p63^{+/+} (β -gal positive) cells while the UGS epithelium consisted of both p63^{-/-} and p63^{+/+} cells (Fig. 3). In the UGS the p63^{+/+} cells were typically found in contact with the basement membrane while the p63^{-/-} cells lined the lumen. The basally located β -gal positive cells were consistently positive for p63, CK5 and CK14 while the β -gal negative p63^{-/-} cells were consistently negative for all three markers (not shown). Interestingly, this layer of p63-positive basal cells was discontinuous in five of the eight p63^{-/-} rescues (Fig. 3b), including three chimeras with no skin or limb abnormalities. This result unequivocally confirmed that these chimeras derived from the p63^{-/-} blastocysts in which the injection of p63^{+/+} ES cells had only partially abrogated the UGS /prostate defects. As a whole, these data confirmed that p63 is necessary for prostate buds formation and demonstrated that p63

expression is required in all the cells that constitute the prostate at this early stage of development.

All the cells that populate the prostate buds at 18.5 dpc display a basal cell phenotype. In fact, prostate secretory cells appear in the prostate epithelium at day 8 after birth (P8). As a result, the analysis of the chimeric embryos does not allow to establish whether p63 is required for the development of secretory cells. Indeed, secretory cells could derive either from p63 positive basal cells within the buds or from p63 negative cells within the UGS epithelium, which subsequently colonize the prostate. To discriminate between these two possibilities, additional blastocyst complementation experiments were performed and the chimeras were analyzed at 7 weeks of age, when the prostate epithelium is fully differentiated. 140 adult chimeras were generated. Six of them presented the pattern of staining characteristic of rescued p63^{-/-} mice by X-gal histochemistry. (Fig. 4c-f). The level of chimerism in organs not depending on p63 for development (e.g. liver and intestinal epithelium) ranged from 40% to 80% in those six animals. One of rescued mice presented minor limb abnormalities including split paws, and digits fusion, indicating incomplete abolishment of the p63^{-/-} phenotype (Fig. 4a, b). The p63^{-/-} genotype of the original blastocyst was further confirmed in the rescued

chimeras by genotyping β -gal negative hepatocytes isolated by laser capture microdissection (not shown).

Preliminary analysis of the prostate of ROSA26 adult mice had demonstrated that β -gal is ubiquitously expressed only in the ventral prostate while subsets of epithelial cells in the dorsolateral and anterior prostate are consistently β -gal negative (Pires and Signoretti, unpublished data). As a consequence, only the ventral prostate was evaluated in our study.



Histochemical analysis of the prostate epithelium of the adult p63^{-/-} rescued mice consistently showed the absence of β -gal negative p63^{-/-} cells (Fig. 4g, i). The prostates of twenty-four control chimeras with comparable level of chimerism (40-85%) were also analyzed. In twenty-three chimeras both basal and secretory cells consisted of both β -gal positive and negative cells (Fig. 4g, i). Only in the control chimera presenting the highest level of chimerism (85%) the prostate epithelium was entirely β -gal positive. The consistence absence of β -gal negative cells in the prostate epithelium of p63^{-/-} rescued mice and the significantly higher frequency of this feature in p63^{-/-} chimeras as compared to control chimeras (Fisher's exact test, $p < 0.0001$) leads to the conclusion that p63 is required for the development of both basal and secretory cells of the prostate. Since p63 is selectively expressed in the basal cell compartment, this result implies that prostate secretory cells derive from p63 positive progenitor basal cells (Fig 1a).

A manuscript describing the results from this study is in preparation.

Meanwhile, we are planning to generate more chimeric mice by utilizing the inverse approach. Specifically, we plan to generate p63^{-/-};p63^{+/+} ROSA26 chimeras by injecting p63^{-/-} ES cells into ROSA26 hemizygous blastocysts. With this novel method, every male chimera that we generate will be informative and will be utilized not only to confirm the results obtained so far but to also study cell lineages in other epithelia expressing p63 (e.g. the bronchial epithelium and the urothelium). This new experimental design will allow us to increase significantly our efficiency in generating informative animals and to completely eliminate the labor-intensive and time-consuming work necessary to identify the p63^{-/-} rescues. To this end p63^{-/-} ES male cells have been generated. Blastocyst injection experiments will be performed shortly.

KEY RESEARCH ACCOMPLISHMENTS

Aim 1

- a. Immunohistochemistry for p63 was performed in the urogenital sinus of 10 wild-type male embryos at 18dpc. Our results demonstrated that during this early stage of prostate development, all the cells in the buds are p63 positive.
- b. We are still working on the optimization of the *in situ* hybridization protocol for the detection of p63 transcripts in skin and prostate tissue sections.

Aim 2

- a. We generated 196 chimeric mice by injecting the selected subclone of ROSA 26 ES cells in blastocysts generated by crossing p63 +/- females with p63 +/- males.
- b. 56 chimeric mice were analyzed at 18.5 dpc and 140 were analyzed when they were 7 weeks old.
- c. We demonstrated that p63 function is cell autonomous.
- d. We demonstrated that skin, limb, and prostate defects in p63 -/- mice can be both partially and completely rescued.
- e. We demonstrated that prostate buds are exclusively populated by ROSA26 cells. This result establishes that p63 is required for the development of all the prostate epithelial cells at this early stage of development.
- f. We showed that 6/6 adult p63 -/- rescued mice but only 1/24 control chimeras consistently showed the absence of β -gal negative p63 -/- cells in the prostate epithelium (Fisher's exact test, $p < 0.0001$). This indicates p63 is required for the development of both basal and secretory cells of the prostate.
- g. A manuscript describing the results of this study is in preparation and will be submitted for publication shortly.
- h. p63 -/- male ES cells have been generated for future experiments.

REPORTABLE OUTCOMES

Manuscripts sponsored by the DADM17-01-1-0051 proposal:

1. Lindeman N, Waltregny D, **Signoretti S**, Loda M. Gene transcript quantitation by real time RT-PCR in cells selected by immunohistochemistry-laser capture microdissection. *Diagn Mol Pathol*. 2002;4:187-92.
2. Weinstein MH, **Signoretti S**, Loda M. Diagnostic utility of immunohistochemical staining for p63, a sensitive marker of prostatic basal cells. *Mod Pathol* 2002;12:1302-1308.
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5. Graner E, Tang D, Rossi S, Weinstein LJ, Lechpammer M, Huesken D, Zimmermann J, **Signoretti S**, Loda M. The isopeptidase USP2a regulates the stability of Fatty Acid Synthase in prostate cancer. *Cancer Cell*. 2004; 5:253-61
6. Murphy M, Carlson JA, Keough MP, Claffey KP, **Signoretti S**, Loda M. Hypoxia regulation of the cell cycle in malignant melanoma: putative role for the cyclin dependent kinase inhibitor p27. *J Cut Pathol*. In Press.
7. Horkan C, Dalal K, Coderre JA, Kiger JL, Dupuy DE, **Signoretti S**, Goldberg SN. Tumor ablation: reduced tumor growth with combined radiofrequency ablation and radiation therapy in a rat breast tumor model. *Radiology*. In Press.

Abstracts sponsored by the DADM17-01-1-0051 proposal:

1. 9th Prouts Neck Prostate Cancer Meeting, 2002. Invited speaker: The basal cell marker p63 and prostate stem cells.
2. Dana-Farber Cancer Institute. Seminars in Genitourinary Oncology, 2003. Invited speaker: "p63 and prostate stem cells".
3. 5th World Congress on Urological Research, London, UK, 2003. Invited speaker: "Defining progenitor cells in the prostate epithelium".
4. Pires M[#], Lindauer M[#], Horner J, Dhar S, McKeon F, Majumder P, Sellers WR, Kantoff PW, Loda M, **Signoretti S**. Blastocyst complementation of p63^{-/-} mice: a

novel tool to define cell lineages in the prostate epithelium. The second p73-p63 International Workshop. Rome, Italy, 2004.

LIST OF PERSONNEL

1. Sabina Signoretti, M.D. (PI) 55% effort

CONCLUSIONS

We demonstrated that p63 function is cell autonomous and that skin, limb, and prostate defects in p63^{-/-} mice can be rescued by injecting p63^{+/+} ROSA26 ES cells into 3.5 dpc blastocysts. The analysis of rescued p63^{-/-} embryos (18.5 dpc) showed that prostate buds are exclusively populated by p63^{+/+} ROSA26 cells. This result establishes that p63 is required for the development of all the prostate epithelial cells at this early stage of development. In addition, 6/6 adult p63^{-/-} rescued mice but only 1/24 control chimeras consistently showed the absence of β -gal negative p63^{-/-} cells in the prostate epithelium (Fisher's exact test, $p < 0.0001$). This indicates p63 is required for the development of both basal and secretory cells of the prostate. Since p63 is selectively expressed in the basal cell compartment, this result implies that prostate secretory cells derive from p63 positive progenitor basal cells.

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